

ENHANCED PEROXIDE RESISTANCE OF *IN VITRO* MUTAGENIZED FLUORIDE-RESISTANT *Klebsiella pneumoniae* UREASES FOR CATALYTIC BUFFERING OF AGENT DECONTAMINATION REACTIONS

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ABSTRACT

Catalytic buffering is an advanced method of pH control for the enzyme-based Advanced Catalytic Enzyme System (ACES) and oxidative surety agent decontamination technologies. Ammonia production from urea by urease neutralizes the production of *O*-alkylphosphonic acids resulting from the hydrolysis of Nerve agents such as Sarin and VX. Fluoride production from Sarin hydrolysis inhibits native urease at low mM concentrations. Hydrogen peroxide, used in oxidative nerve agent treatment, is also potent inhibitor of urease activity. Fluoride-resistant (FR) ureases developed previously in our laboratory were >95% inhibited by 1% hydrogen peroxide. To overcome this problem, the FR mutant urease structural genes of *Klebsiella pneumoniae* were mutagenized *in vitro* in an *E. coli* mutator strain to produce ureases with superior peroxide-resistance (PR). Mutagenized *ureABC* plasmids were recovered and co-transformed into an *E. coli* catalase mutant (UM2) with a plasmid bearing the urease accessory genes (*ureDEFG*) needed for nickel incorporation. The double-plasmid recombinant colonies were immobilized on nylon, lysed and screened *in situ* for peroxide-resistant urease activity. Several PR clones were isolated from this procedure with enhanced urease activity in 1% hydrogen peroxide. Several generations of *in vitro* mutagenesis of the PR *ureABC* plasmids resulted in ureases with many times the activity of their FR progenitors in 1% peroxide. These results show the promise of mutant peroxide-resistant ureases as catalytic buffering catalysts for agent decontamination reactions.

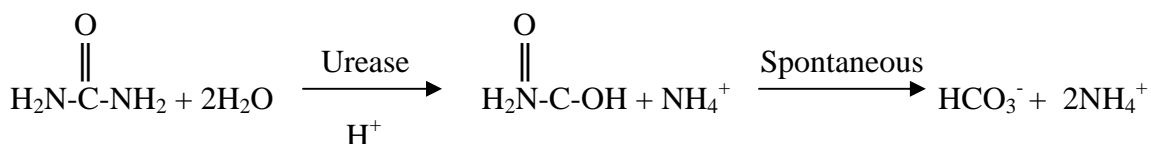
INTRODUCTION

Catalytic buffering is an advanced pH control method. It relies on a catalyst, e.g. enzyme, to produce ions from a substrate to regulate the pH of the solution in question.

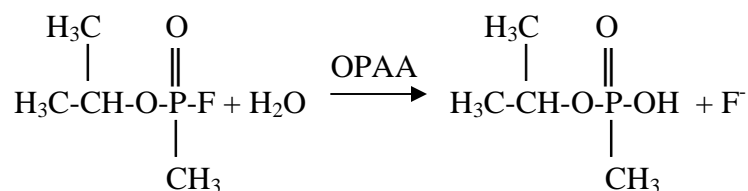
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Catalytic buffering is particularly attractive to the Advanced Catalytic Enzyme System (ACES) because the acidic *O*-alkylphosphonate (EMPA, IMPA, etc.) products of the OPAA- and OPH-mediated G and V agent hydrolysis dramatically lower the reaction pH. Catalytic buffering creates an on-demand neutralization system without the use of additional harsh chemicals to maintain the desired reaction pH for agent detoxification enzymes.

An example of a possible catalytic buffering system is ammonia production resulting from the hydrolysis of urea by Urea Amidohydrolase (Urease):



Urease is nickel metalloenzyme widely distributed in nature¹. Three structural genes (*ureABC*) code for the protein subunits but activity requires four accessory genes (*ureDEFG*) for nickel uptake and incorporation². Urea is a neutral molecule that does not affect the pH of the aqueous solution. Ammonia production by urease raises the solution pH. This rise in pH produced by urease activity counters the pH decrease arising from the *O*-alkylphosphonate products of OPAA or OPH catalysis of organophosphorus nerve agents. While this seems an appropriate enzyme for catalytic buffering, urease is inhibited by low levels of fluoride ion³ and peroxide. Fluoride ion is one of the products of GB hydrolysis:



Previous efforts in our laboratory produced mutant fluoride-resistant (FR) ureases with reduced fluoride inhibition relative to the native *Klebsiella aerogenes* enzyme⁴. FR mutant and native microbial urease testing showed that although the mutant urease was less susceptible to hydrogen peroxide inhibition than the native enzyme, 1% peroxide inhibited the enzyme > 90%.

To address the urease peroxide sensitivity, efforts were initiated to produce peroxide-resistant ureases from the FR ureases. The multigenerational *in vitro* random mutation procedure used to produce the FR ureases was modified to produce peroxide resistant (PR) ureases. The solid phase screening procedure was adapted to accommodate peroxide inhibition assays. This report outlines these modifications and the development and analysis of PR KAU ureases.

MATERIALS AND METHODS

Plasmids p9-1 and pKAUDEFG were previously described⁴. *E. coli* strains used in these experiments were DH10B (Invitrogen), XL1-red (Promega) and UM2 (*E. coli* Genetic Stock Center, Yale University). The plasmid cloning host was DH10B;

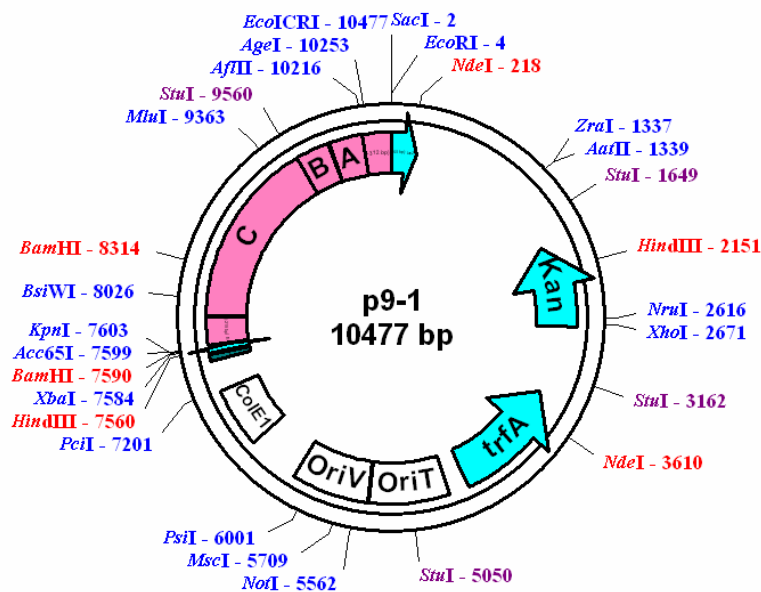


Figure 1. p9-1 Plasmid Map

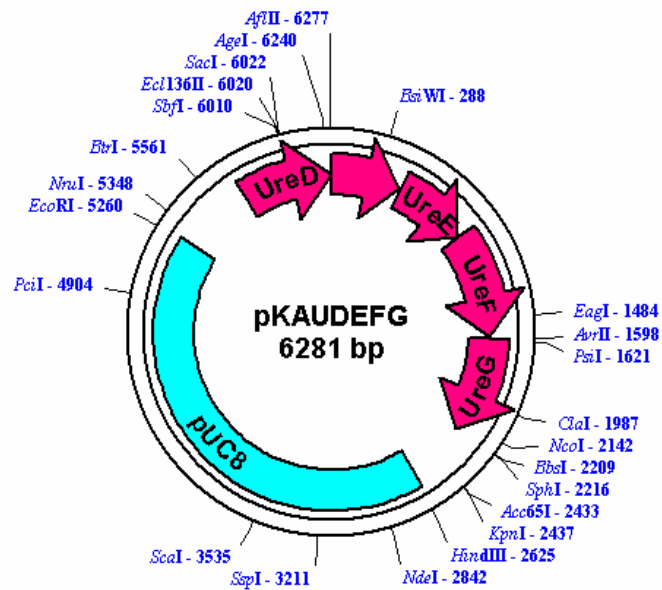


Figure 2. pKAUDEFG plasmid map.

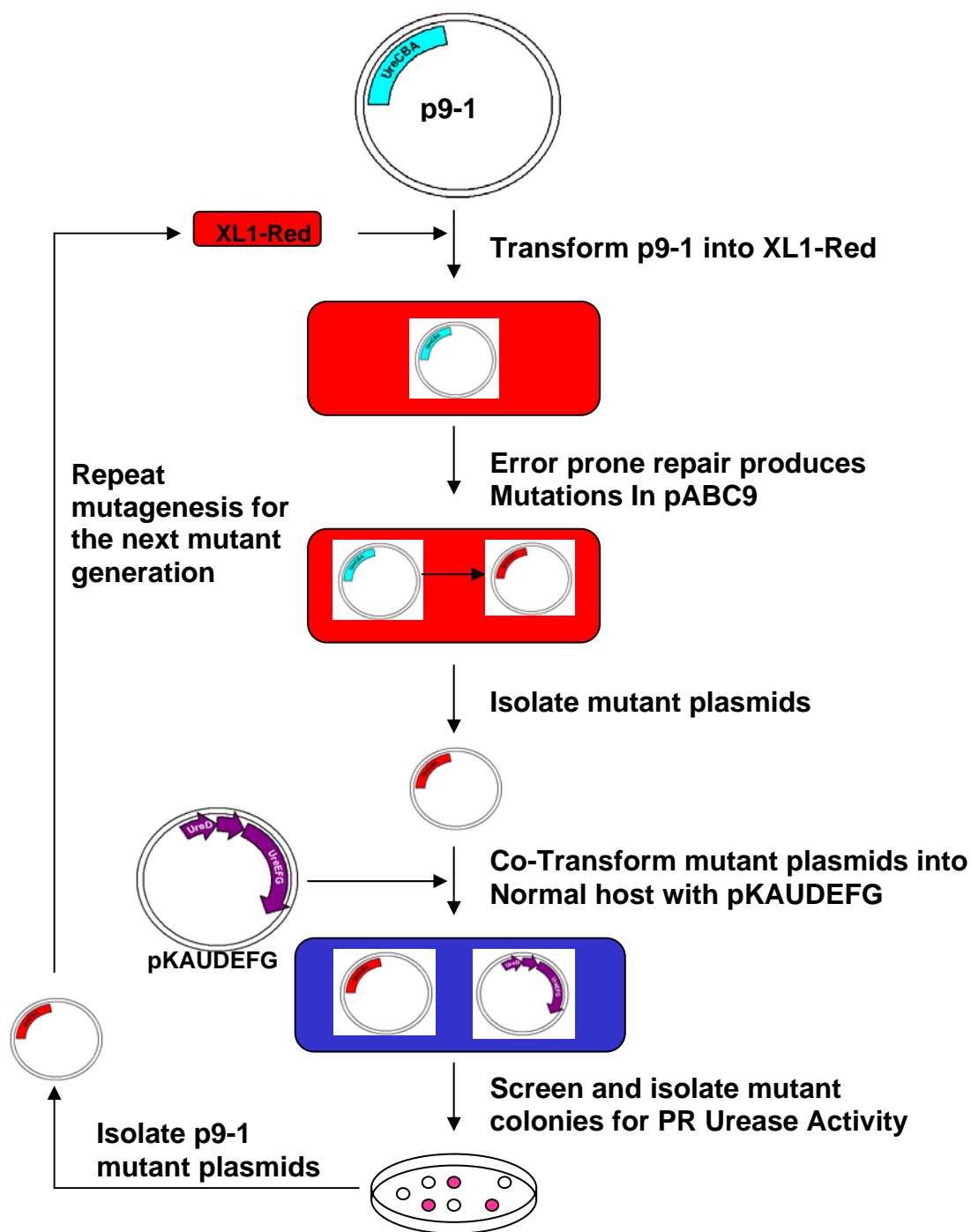


Figure 3. *In vitro* mutagenesis of the KAU structural gene cluster

XL1-Red was used for mutagenesis only. UM2 is double catalase mutant (katE2, katG15⁵) used for peroxide resistance screening. p9-1 contains the FR mutant KAU structural genes⁴ cloned into the broad host range cloning vector, pCM66⁶. Growth media were Luria agar (LA), Luria agar/broth + 0.4 mM NiCl₂ (LA_{nick} or LB_{nick}). Recombinant

clone selection was 50 µg/ml ampicillin (pKAUDEFG) and/or 25 µg/ml kanamycin (p9-1-derived plasmids) or both. Addition of Bluogal to LA plates provided blue/white color screening needed for recombinant plasmid identification. Screening filters were 82 mm Biotary B circles. Screening pads were VWR 283 (Ahlstrom) cut to size. Rubbermaid supplied the large polycarbonate screening boxes (18 X 26 X 3 ½") for the mass screening.

In vitro Plasmid Mutagenesis

Transformation of urease structural gene plasmids into XL1-Red competent cells (Stratagene) resulted in random mutations after incubation for 48 h at 37°C. The plasmids were recovered from the colonies scraped off the plates and the plasmid pool purified with the Wizard SV procedure (Promega).

Screening Mutagenized p9-1

Mass Initial Screening:

The mutagenized plasmids were co-transformed with pKAUDEFG into Electrocompetent UM2, diluted and spread onto numbered LA_{nick} Kan₂₅ Amp₅₀ plates. The colonies were lifted from the plates with numbered Biotary B filters after overnight incubation. The filters were placed colony side up into the bottom of water-dampened glass Petri dishes. Colonies re-grew on the lifted plates after overnight incubation at 37°C. The colonies on the filters were lysed with chloroform in the lids of inverted glass Petri dishes for 30'. The lids were removed from the plates and the residual chloroform evaporated for 2' in a fume hood. The filters were laid on pads soaked with pre-inhibition solution (0.5 mM HEPES, pH 7, 0.1 M sodium fluoride) for 10', RT in a large screening box. After pre-inhibition, the filters were transferred to pads soaked with the assay solution (1-2 % Hydrogen peroxide, +/- 0.1 M sodium fluoride in 0.5-1.25 mM HEPES, pH 7, 55 µg/ml Phenol Red, 10 mM Urea) in another large screening box. The pink color development was noted at RT from 30" to 10'. The plate number and location of the Lysed, Ure⁺ positive colonies were used to find the live colonies on the re-grown original plates.

Patch Screening:

After the colonies re-grew on the lifted plates from above, the positive colonies were picked and patched onto LA_{nick} Kan₂₅ Amp₅₀ plates. The patched plates were incubated overnight at 37°. The original patch plate was replica plated with velveteen onto four LA_{nick} Kan₂₅ Amp₅₀ plates. After overnight growth, the replicated patches were lifted from plates with filters, lysed and assayed in the same manner described above but assayed in individual polystyrene plates instead of the large box. The assay solution used for this analysis varied in the peroxide (1-3%) and in the buffer (0.5-5 mM HEPES) concentration to detect the best PR activity of the patched clones. Clones with the best activity in the highest buffer/peroxide concentration were picked from the original patch plate, inoculated into 5 ml of LB_{nick} Kan₂₅ Amp₅₀ and incubated at 37°C overnight. This culture served as the source for the inoculum for the crude extract preparation, the frozen stock and the mini plasmid prep.

Intracellular Extract Preparation

0.5 ml of the 5 ml LB culture from above was inoculated into 100 ml of LB_{nick} Kan₂₅ Amp₅₀. This culture incubated at for 24 h, 220 rpm, 37°C. The cells were harvested, washed once in 0.5 mM HEPES, pH 7, 4°, and resuspended in 0.5 ml of the same buffer. The cells were broken in a Mini-Bead Beater (Biospec) and the intracellular extract clarified at 20,000 X g at 4°, 5'. A repeat of the clarification process removed traces of cells and glass beads.

Intracellular Extract Analysis

Protein content of the intracellular extract was determined with the Coomassie Blue Plus reagent (Pierce). Intracellular extracts assayed for urease activity in 1% peroxide using the Nessler's reagent procedure to detect ammonia from the time-sampled reaction. Enzyme samples were pre-incubated for 10' in the peroxide reaction prior to initiating the reaction with urea. Reaction was 10 mM Urea, 5 mM HEPES, pH 7 +/- 1% peroxide. Nessler's ammonia in the time samples was quantified from ammonium chloride standards. Specific activity (SA) reported as $\mu\text{moles Urea hydrolyzed/min-mg protein}$ in the sample. Data plotted in Excel.

Cloning Procedures

Molecular biology manipulation, transformations, etc., were described previously⁴.

RESULTS

Screening Double Plasmid Clones for PR Urease Activity in UM2

Although solid phase screening procedures were established for the detection of FR urease mutants, these procedures required alteration to accommodate PR urease screening. First, DH10B was substituted with the catalase-deficient host *E. coli* (UM2) because the catalase and peroxide reaction produced foam on the screening filters, obscuring the results. The gas bubbles formed from this reaction also interfered with spectrophotometric assays. The catalase-deficient UM2 does not form bubbles or foam from hydrogen peroxide. Substitution of Biodyne B nylon for Nitrocellulose filters in the solid phase screening prevented any potential reactions of an oxidizer (peroxide) with nitrocellulose. Inclusion of NaF in the screening reaction assured that some degree of fluoride resistance would remain in the PR mutants selected from the screening procedure. The 9-1 FR mutant was the only pABC9 FR mutant that showed sufficient activity in UM2 to monitor the peroxide-inhibited reaction with the Nessler's reaction. Urease expression in the UM2 host produced urease with ~1/10 the activity seen in other *E. coli* hosts. Another higher expressing construct of the native urease, pDABC5, was

needed to produce sufficient activity for detection in UM2. The cause of low UM2 urease activity is unknown.

PR Mutant Production

Several mutants with higher activity than 9-1 in 1% peroxide were produced and characterized (Table 1). The best mutants, 17-2 and 26-2, showed 4-8X the urease activity of 9-1 in intracellular extracts in 1% peroxide, respectively. When assayed with combined 1% peroxide/50 mM NaF, the specific activity was not significantly different from that observed with NaF alone.

TABLE 1
Intracellular Extract Urease Activity of *in vitro* Mutants in Peroxide and Fluoride.

Mutant Extract	No P/F	1% P	0.05 M F	0.05M F+1% P
Native (pDABC5)	1.86	0.007	0	ND
9-1 (parent)	2.46	0.118	0.01	ND
16-1RC	1.89	0.159	0	ND
26-2 (2 nd gen.)	14.99	0.909	0.024	0.036
17-2 (2 nd gen.)	31.46	0.498	0.037	0.038
26-3 (2 nd gen.)	8.16	0.811	0.003	ND
26-1A (2 nd gen.)	10.13	0.41	0.016	ND
18-1 (2 nd gen.)	18.59	0.317	0.019	ND
19-3 (2 nd gen.)	9.62	0.237	0.022	ND
19-1 (2 nd gen.)	16.21	0.227	0.015	ND
26-3 (1 st gen.)	8.16	0.811	0.003	ND
26-1 (1 st gen.)	10.13	0.407	0.016	ND

Specific activity (SA) expressed as μ moles Urea hydrolyzed/min-mg protein.

Note: All but the native, pDABC5 extract were mutants derived from the original pABC9 plasmid. Expression of urease is considerably higher in pDABC5 than in pABC9. Expression of pABC9 in UM2 gave cell extract urease activity too low to measure.

ND = Not determined.

CONCLUSIONS

In vitro mutagenesis of FR p9-1 combined with lysed colony filter screening of the KAU *ureABC* structural genes was successfully used to produce and identify peroxide-resistant (PR) mutant ureases. Some of these mutants still retained fluoride resistance (TABLE 1). The best peroxide-resistant activity was 4-8X higher than that seen in the 9-1 parent and 70-125X that seen in the native KA urease.

As previous work with the fluoride-resistant (FR) mutant ureases showed that these enzymes could be used successfully to catalytically buffer OPAA reactions with

high DFP, it is anticipated the PR mutant ureases resulting from this project will show similar success in the oxidative reactions.

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